β3-Adrenoceptor detection and signal transduction: focus on antibody validation and urinary bladder

Cernecka, Hana

CHAPTER 3

Specificity evaluation of antibodies against human β_3-adrenoceptors

Hana Cernecka, Peter Ochodnicky, Wouter H. Lamers, Martin C. Michel

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ABSTRACT

β_3-Adrenoceptors are a promising drug target for the treatment of urinary bladder dysfunction but knowledge about their expression at the protein level and their functional role is limited, partly due to a lack of well validated tools. As many antibodies against G-protein-coupled receptors including those against β_3- and other β-adrenoceptor subtypes lack selectivity for their target, we have evaluated the specificity of five antibodies raised against the full length protein of the human β_3-adrenoceptor (H155-B01), its N-terminus (LSA4198, TA303277) and its C-terminus (AB5122, Sc1472) in immunoblotting and immunocytochemistry. Our primary test system were Chinese hamster ovary cells stably transfected to express each of the three human β-adrenoceptor subtypes at near physiological levels (100-200 fmol/mg protein). None of the five antibodies exhibited convincing target specificity in immunoblotting with Sc1472 apparently being least unsuitable. In immunocytochemistry LSA4198 and Sc1472 appeared most promising, exhibiting at least some degree of specificity. As these two antibodies have been raised against different epitopes (N- and C-terminus of the receptor, respectively), we propose that concordant staining by both antibodies provides the most convincing evidence for β_3-adrenoceptor labeling in cyto- or histochemistry studies.

INTRODUCTION

Based upon effects of β_3-adrenoceptor agonists with variable degrees of specificity, β_3-adrenoceptors are believed to play a physiological role in various tissues including the central nervous system, heart, at least some vascular beds, uterus, urinary bladder, liver and adipocytes, although some of these effects may largely be restricted to rodents (Ursino et al., 2009; Michel et al., 2010). Based on such findings, β_3-adrenoceptor agonists were originally under consideration for the treatment of diabetes mellitus type 2 (Grujic et al., 1997) and obesity (Arch et al., 1984). Despite remarkable effects on adipose tissue lipolysis, metabolic rate and insulin sensitivity in rodents, β_3-adrenoceptor agonists have either been of limited efficacy (Danforth and Himms-Hagen, 1997; Arch and Wilson 1996; Arch 2008) or their activities have been accompanied by significant side effects (Connacher et al., 1990) in humans. Based on *in vitro* or rodent *in vivo* findings possible therapeutic roles for β_3-adrenoceptor ligands has been proposed in anxiety and depression (Stemmelin et al., 2008), heart failure (Rasmussen et al., 2009), premature labor (Croci et al., 2007) and retinal disease (Steinle et al., 2003; Mori et al., 2010) but these roles remain to be substantiated by clinical data. In contrast, β_3-adrenoceptor agonists including mirabegron (formerly known as YM-178) have not only shown promise in animal models of bladder dysfunction and human bladder *in vitro* (Michel et al., 2011b) but also shown beneficial results in randomized, double-blind, placebo-controlled clinical studies (Khullar et al., 2011; Nitti et al., 2011).

Progress in the mechanistic understanding of desirable and untoward effects of β_3-adrenoceptor agonists in humans has been hampered by a lack of suitable tools to explore their expression pattern at the protein level (Michel et al., 2011a). Thus, the expression pattern and tissue functions of β_1- and β_2-adrenoceptors have been well characterized, but those of β_3-adrenoceptors remain ill defined (Michel et al., 2010). The only human tissue for which a large body of data exists on β_3-adrenoceptors is the urinary bladder (Michel and Vrydag, 2006;

Yamaguchi and Chapple, 2007). Progress in the identification of β_3-adrenoceptor protein in other tissues is hampered by a lack of suitable tools such as radioligands or antibodies (Vrydag and Michel, 2007). Specifically, it has been shown that antibodies against β_3-adrenoceptors (Pradidarcheep et al., 2009), other β-adrenoceptor subtypes (Hamdani and van der Velden, 2009) or other G-protein-coupled receptors in general (Michel et al., 2009) in many if not most cases lack selectivity for their cognate receptors when tested with stringent criteria. On the other hand, other data have indicated that some β_3-adrenoceptor antibodies may be selective for their target receptor (Bundgaard et al., 2010; Chamberlain et al., 1999; Guillaume et al., 1994; Kullmann et al., 2009; Limberg et al., 2010), but in most cases the degree of validation has remained limited. Against this background we have systematically tested a range of antibodies raised against different epitopes of the human β_3-adrenoceptor for their target selectivity.

MATERIAL AND METHODS

Cell lines expressing adrenoceptors. Chinese hamster ovary (CHO) cells stably transfected with human β_1-, β_2- or β_3-adrenoceptors or empty vector were obtained from Dr. Carsten Hoffmann (Würzburg, Germany) and cultured in Dulbecco's Modified Eagle's Medium with nutrient mixture F12 in the presence of 10% foetal bovine serum, 100 U/mL penicillin G, 100 μg/ml streptomycin, 2 mM L-glutamine and, to maintain selection pressure, 0.2 mg/ml geneticin as previously described (Niclauß et al., 2006). Of note, these cell lines do not to over-express the receptor subtypes but rather express about physiological expression levels, i.e. approximately 100-200 fmol/mg protein as determined by radioligands studies (Hoffmann et al., 2004; Niclauß et al., 2006). UROtsa cells, which express β_3-adrenoceptor mRNA (Ochodnicky et al., 2012) and were shown to respond functionally to some β_3-adrenoceptor agonists (Limberg et al., 2010), were maintained in RPMI medium supplemented with 5% foetal bovine serum, 1% sodium pyruvate, 100 U/ml penicillin G and 100 μg/ml streptomycin.
Western blot analysis. Cell monolayers were washed with phosphate-buffered saline (PBS) and detached by scraping. Cells were collected by centrifugation and homogenized with an Ultra-Turrax T8. Cell membranes were collected by further centrifugation at 50,000 g for 22 minutes at 4ºC. The resulting pellets were dissolved by homogenization in TED-buffer (10 mM Tris-HCl, 1.5 mM EDTA and 0.5 mM dithiothreitol (Sigma Aldrich, Zwijndrecht, Netherlands), pH 8.0). As positive control for Western blot analysis, 0.1 g of frozen mouse heart was homogenized in buffer containing 150 mM NaCl, 1% TritonX-100, 50 mM Tris-HCl (pH 8.0) and protease inhibitor cocktail kit 100x (#78410; Thermo Scientific, Delft, Netherlands). The homogenate was centrifuged at 8,800 g for 10 minutes and the supernatant taken. The total protein concentration was determined according to Bradford (1976).
Each sample containing 15, 20 or 50 μg of total protein was subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis on a 4-12% gel under reducing conditions and transferred to nitrocellulose membranes. Membranes were blocked for 2 hours with different concentrations (1, 5 or 10%) of non-fat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TTBS). After blocking, membranes were incubated with various anti-human β_3-adrenoceptors primary antibodies at the concentrations described in the Table 1 at 4 ºC overnight. After two washes with TTBS, the blots were incubated with appropriate secondary

horseradish peroxidase-conjugated antibody in TTBS with 5% milk powder (Table 1) for 1 hour at room temperature. Immunoreactive proteins were detected by chemiluminiscence (Lumi-Light ^{PLUS} Western Blotting Substrate, #12015196001, Almere, Netherlands).

Immunocytochemistry. CHO cells stably expressing human β_1-, β_2- or β_3-adrenoceptors and CHO cells stably transfected with empty vector were plated in 16-well or 96-well plates coated with collagen IV (Sigma Aldrich, Zwijndrecht, Netherlands). Three different fixatives were tested to find the optimal conditions for antibody specificity (ice-cold 2:2:1mixture of methanol-acetone-water, 3.6% formaldehyde in PBS). Based on initial experiments, fixation with 3.6% formaldehyde was selected for all further experiments. After 5 minutes of fixation, cells were permeabilized with the 0.1% TritonX-100 (Sigma Aldrich, Zwijndrecht, Netherlands) in PBS for 10 minutes. Subsequently, cells were blocked with 1% bovine serum albumin (BSA) in PBS for 30 minutes and incubated overnight at 4ºC with primary antibody (Sc1472 or LSA4198) in PBS containing 0.1% BSA. Different dilutions of antibody were tested. The best results were observed with dilution 1:500 for Sc1472 and 1:400 for LSA1498. After washing with PBS, cells were incubated with alkaline phosphatase-conjugated secondary antibody or Alexa488-conjugated secondary antibody (Table 1) for 1 hour at room temperature. Staining of alkaline phosphatase-conjugated secondary antibody was developed with NBT/BCIP Stock Solution (Roche Applied Science, Mannheim, Germany) in 0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 0.05 M $MgCl_2$ solution, which produced a dark-blue staining. Images were taken at 10x magnification using a Nikon Eclipse TE2000-U fluorescence microscope (Plan Fluor ELWD 20x objective, Nikon DXM1200F digital camera) with NIS Elements AR 2.30 software.

Data presentation. All data shown are from a representative experiment which was performed at least twice with similar results.

RESULTS

The LSA4198 antibody, directed against the N-terminus of the human β_3-adrenoceptor (Table 1) did not label any specific band in Western blots of membrane extracts from CHO cells transfected with the human β_3-adrenoceptor as compared to those transfected with human β_1- or β_2-adrenoceptors; moreover, no specific band of expected molecular weight (44 kDa) was observed in mouse heart (Figure 1A). In agreement with previous findings (Kullmann et al., 2009; Limberg et al., 2010), immunocytochemical staining with LSA4198 was observed in CHO cells stably expressing human β_3-adrenoceptor, whereas less staining was observed in the CHO cells stably transfected with empty vector and CHO cells stably transfected with human β_1- or β_2-adrenoceptors (Figure 1B).

The H155-B01 antibody, raised against the full length protein of the human β_3-adrenoceptor (Table 1), also failed to detect a specific band in CHO cells transfected with the human receptor subtype (Figure 2A). In mouse heart a minor band of approximately 54 kDa and a major band of approximately 70 kDa were detected (Figure 2A).

The AB5122 antibody, raised against the C-terminus of the human β_3-adrenoceptor (Table 1), detected a weak band at approximately 44 kDa in β_3-adrenoceptor-expressing CHO cells, which was more prominent than in cells expressing β_1- or β_2-adrenoceptors (Figure 2B). However, this band was one of the weaker ones in the overall blot. Based on the results with

our standard approach with 5% of non-fat dry milk in TTBS as the blocking solution and a 1:500 dilution of the antibody, we also performed experiments with a 1:200 dilution of the antibody. This increase in antibody concentration, however, did not llnprove the detected signal (data not sho\Vll).

Table 1: Supplier information on antibodies stated to be specific for human β_3-adrenoceptors being tested in present study. All information in this table is based upon information provided by the respective manufacturers. Abbreviations: IB, immunoblot; IHC, immunohistochemistry; IF, immunofluorescence; AP, alkaline phosphatase; HRP, horseradish peroxidase; H, human; Rb, rabbit; M, mouse; G, goat.

Antibody	Supplier	Reactive/ Host Species	Epitop	Dilution IB	Blocking IB	Dilution ICC	Blocking ICC	Claimed uses	Protein concentration	
									CHO cells	mouse heart
LSA4198	MLB Intern.	H/Rb	N-terminus	1:200 in 5% TTBS	5% milk	1:400 in 0.1% BSA	1% BSA	IHC	20µg/ 20µl	15µg/ 20µl
H155-B01	Abnova	H/M	Full length protein	1:1000 in 5% TTBS	5% milk	-	-	IB, IHC, IF	20µg/ 20µl	15µg/ 20µl
AB5122	Chemicon Intern.	H/Rb	C-terminus	1:200 in 5% TTBS	5% milk	-	-	IB, IHC, ELISA	70µg/ 20µl	15µg/ 20µl
TA303277	Origene	H/G	N-terminus	1:5000 in 1% TTBS	1% milk	-	-	IB, ELISA	40µg/ 20µl	15µg/ 20µl
Sc1472	Santa Cruz	H/G	C-terminus	1:200 in 5% TTBS	5% milk	1:500 in 0.1% BSA	1% BSA	IB, ELISA, IF	50µg/ 20µl	15µg/ 20µl

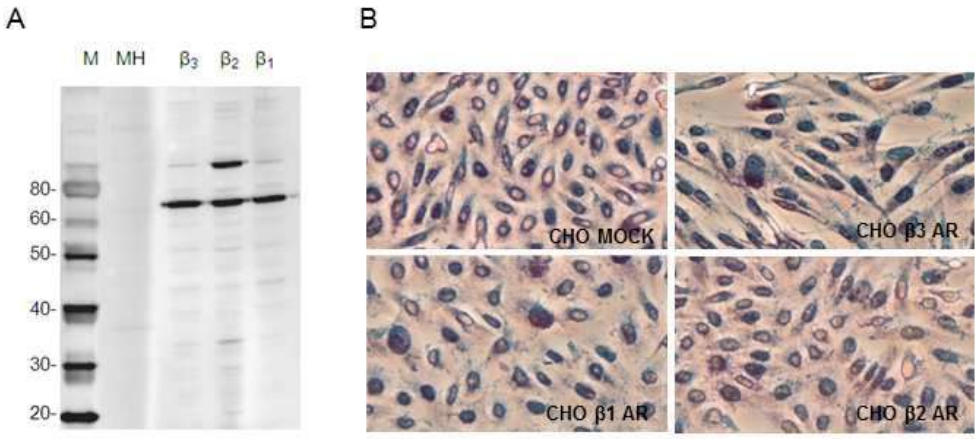


Figure 1: Immunoblot (panel A) and immunocytochemistry (panel B) with antibody LSA4198 in CHO cells stably expressing human β_1-, β_2- or β_3-adrenoceptors. M: molecular weight markers. Data are from a representative experiments performed at least twice with similar results.

The TA303277 antibody raised against N-terminus of the human β_3-adrenoceptor (Table 1) failed to detect a specific band in CHO cells stably transfected with human β_3-adrenoceptor (Figure 2C). In mouse heart we detected two major bands of approximately of 38 kDa and 70 kDa and two minor bands of 44 kDa and 50 kDa (Figure 2C).

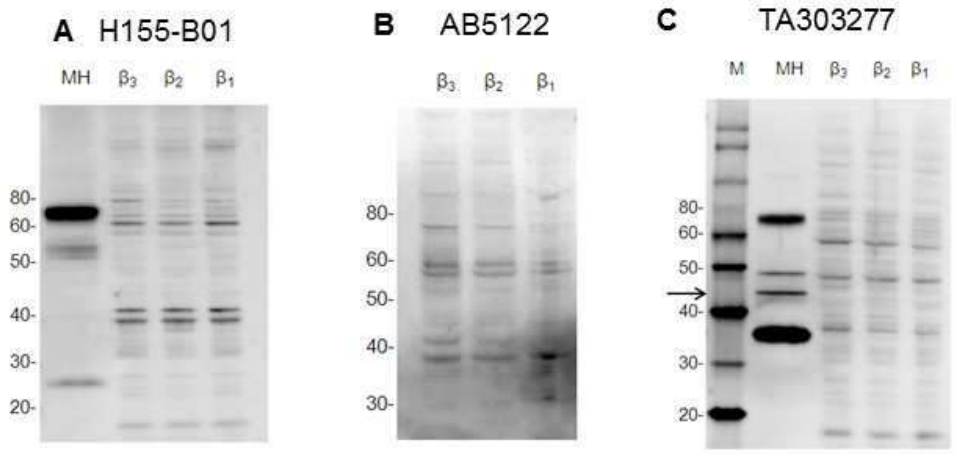
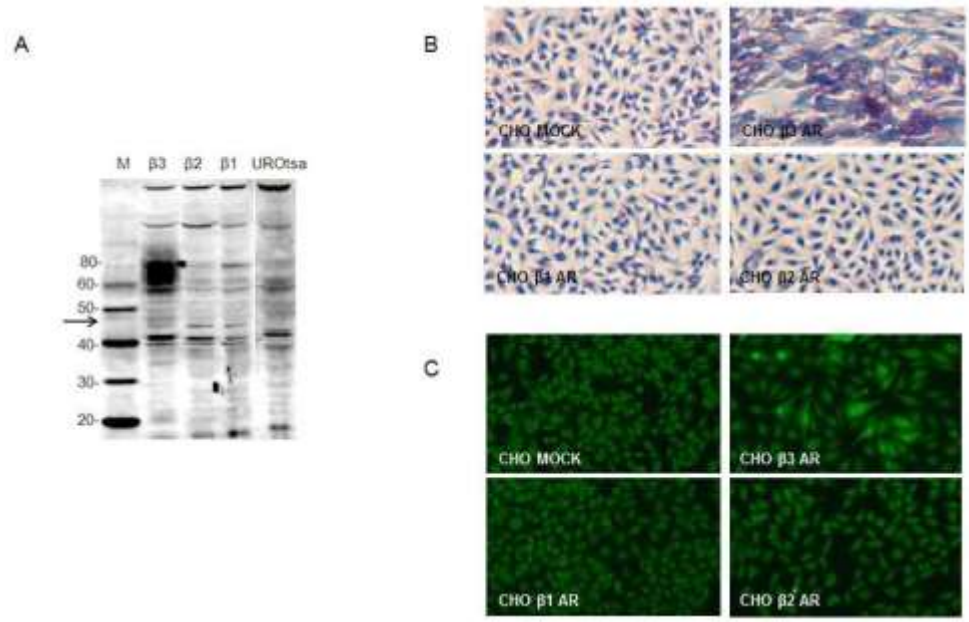


Figure 2: Immunoblot with antibodies H155-B01 (panel A), AB5122 (panel B) and TA303277 (panel c) in CHO cells stably expressing human β_1-, β_2- or β_3-adrenoceptors and in mouse heart (MH). M: molecular weight markers. Data are from a representative experiments performed at least twice with similar results.

The Sc1472 antibody, directed against the C-terminus of the human β_3-adrenoceptor (Table 1), detected several bands in β_3-transfected CHO cells (Figure 3A). One of them was seen at an apparent molecular weight of 44 kDa and was more prominent in cells expressing β_3- than those expressing β_1- or β_2-adrenoceptors. This band was also detected in UROtsa cells (Figure 3A). Another band with an apparent size of 70 kDa was only seen in the β_3-adrenoceptor-expressing cells, and a band of that size had previously been described as a glycosylated form of the β_3-adrenoceptor (Tate et al. 1991).

In immunocytochemistry performed with Sc1472 and secondary antibody conjugated with alkaline phosphatase, a high level of specific staining was observed in CHO cells stably expressing human β_3-adrenoceptor as compared to the CHO cells stably transfected with empty vector or with human β_1- or β_2-adrenoceptors (Figure 3B). Experiments with a fluorescent secondary antibody yielded a brighter stain in β_3-adrenoceptor-transfected cells but that stain appeared to be largely localized to the nucleus (Figure 3C).



<u>Figure 3:</u> Immunoblot (panel A), immunocytochemistry with enzymatic detection (panel B) and immunocytochemistry with fluorescent detection (panel C) with antibody Sc1472 cells stably expressing human β_1-, β_2- or β_3-adrenoceptors and in UROtsa cells. M: molecular weight markers. Data are from a representative experiments performed at least twice with similar results.

DISCUSSION

A monoclonal antibody against the human the β_3-adrenoceptor had been reported in the past (Chamberlain et al., 1999) and subsequently used by other investigators (Perfetti et al., 2001; De Matteis et al., 2002). This antibody had been validated in immunoblot of CHO cells transfected with each of the three human subtypes, by flow cytometry where the detected signal was quantitatively related to receptor expressed as detected by radioligand binding, and by deglycosylation experiments. Unfortunately, this antibody has never been commercialized and is no longer available from either the original investigators or those who have obtained it from them in the past. Therefore, present investigators primarily turn to commercially available β_3-adrenoceptor antibodies. While all of them represent polyclonal antibodies, they have been raised against different portions of the receptor or related peptides and in different species (Table 1). Therefore, the present study was designed to test and validate several of these antibodies against human β_3-adrenoceptors, focusing on those which had been used in published reports by other investigators.

Methodologicalconsiderations

Our primary model system were CHO cells stably transfected with the receptor subtypes or empty vector, an approach also used by others exploring β_3-adrenoceptor antibodies (Kullmann et al., 2009; Limberg et al., 2010;Pradidarcheep et al., 2009).

In many if not most cases, stably transfected cells express very high numbers of receptors, often several pmoles/mg protein, while native tissue expression of most receptors is a few hundred fmoles/mg protein or less. If a given antibody has a limited degree of selectivity for its cognate receptor but also binds to some other targets, a very high expression level will boost the "specific" signal and lead to an overestimation of selectivity as compared to what can be observed with much lower expression densities observed in native tissues. To avoid this potential limitation, we have chosen for CHO cells expressing only 100-200 fmol/mg protein for each of the three β-adrenoceptor subtypes (Hoffmann et al., 2004; Niclauß et al., 2006).

On a more limited scale we have used two sources natively expressing β_3-adrenoceptors, i.e. the human urothelium-derived UROtsa cells (Limberg et al., 2010; Ochodnicky et al., 2012) and mouse heart (Brixius et al., 2006; Bundgaard et al., 2010). The latter was used based on the possibility that a given antibody targeted at the human receptor may cross-react with its murine ortholog, as has been proposed in the past for one of the commercially available β_3-adrenoceptor antibodies (Kullman et al., 2009). In this regard, a positive finding of a single band of appropriate size in mouse heart homogenates could support specificity claims for a given antibody, but a negative result is difficult to interpret as even an antibody with subtype specificity for the human β_3-adrenoceptor may not recognize its murine ortholog.

Of note, the selectivity of a given antibody in one application, e.g. immunoblotting, cannot necessarily be seen as evidence for selectivity and usefulness in another application, e.g. immunocyto- or histochemistry (Skliris et al. 2002), and some commercial suppliers claim only some applications for their antibodies (Table 1).

Moreover, the apparent specificity of an antibody in immunohistochemistry may be affected by a range of experimental details (Petrusz et al. 1976), and specifically fluorescence-based detection methods may be sensitive to this (Lorincz and Nusser, 2008). Based on these

considerations our antibody evaluation has primarily been based on immunoblotting, but immunocytochemistry has additionally been applied in some cases.

Evaluationofspecificantibodies

Using immunocytochemistry in CHO cells transiently over-expressing all three β-adrenoceptor subtypes it has previously been shown that antibody LSA4198, directed against the N-terminus of the human β_3-adrenoceptor (Table 1), exhibits specific β_3-adrenoceptor staining (Kullmann et al., 2009; Limberg et al., 2010). Our experiments confirm some degree of specific β_3-adrenoceptor staining by LSA4198, although the degree of specificity appeared to be somewhat less than in the previous studies. This difference can potentially be explained by the fact that Kullmann et al. and Limberg et al. have used overexpression of receptors, whereas we have used cells with an expression level assumed to reflect physiological receptor density. By amplifying the "specific" without changing the "non-specific" signal, cells overexpressing the target receptor may overestimate the degree of specificity of a given receptor. Kullmann et al. (2009) also showed apparently specific staining of β_3-adrenoceptors in rat bladder urothelium, indicating that this receptor may cross-react with the rodent ortholog of the subtype. Moreover, the staining in rat urothelium was qualitatively very similar to that obtained with an antibody directed against the rat β_3-adrenoceptor within the same study and with another antibody raised against the rat receptor in our hands (unpublished findings). In contrast, this antibody did not exhibit any specificity in our immunoblot experiments with the human receptor subtypes and also did not detect a specific band in a mouse heart extract. Therefore, in line with the recommendation by its manufacturer (Table 1) this antibody could be useful for immunocyto- and histochemistry applications with human and apparently also rat β_3-adrenoceptors. Accordingly, the LSA4198 has successfully been used to detect β_3-adrenoceptors by immunohistochemistry in the human bladder (Limberg et al., 2010).

The H155-B01 antibody, raised against the full length protein of the human β_3-adrenoceptor, similarly lacked selectivity for human β_3- over β_1- or β_2-adrenoceptors in immunoblotting. While only two major bands were seen in mouse heart, neither of them corresponded to the reported size of the rodent receptor in its native or glycosylated form (Bundgaard et al., 2010). Of note, the antibody was produced in mice and, therefore, might detect murine receptor as there is a high homology between the human and mouse β_3-adrenoceptors (Strosberg and Pietri-Rouxel, 1996). However, the manufacturer does not claim this antibody to detect rodent β_3-adrenoceptors, and according to our data it is also unsuitable for the detection of the human subtype in immunoblotting.

The AB5122 antibody, directed against C-terminus of the human β_3-adrenoceptor, exhibited a similar staining pattern as the LSA4198 antibody in immunoblots. While a band with a molecular weight of 44 kDa was observed in CHO cells transfected with β_3-adrenoceptors, this band was also found in β_1- and β_2-adrenoceptor-transfected cells. This antibody was used by another research group to investigate expression of β_3-adrenoceptors in myometrium of nonpregnant and pregnant women (Rouget et al. 2005). Unlike our results, they found a β_3-protein at 68 kDa, using CHO cells transfected with the human β_3-adrenoceptors as a positive control. Other investigators reported a 60 kDa band with this antibody in human bronchi (Bossard et al. 2011). A 60 kDa band was also observed in our CHO cell experiments, but was

similarly detected in cells expressing β_1- or β_2-adrenoceptors. In the absence of other data, we feel that this antibody is insufficiently validated to justify its use.

The TA303277 antibody, directed against N-terminus of human β_3-adrenoceptors, did not specifically label any band in CHO cells stably transfected with human β_3-adrenoceptor. Several bands were observed in mouse heart, but none of them corresponded to that reported as being specific based on knock-out mice (Bundgaard et al., 2010). Hence, this antibody also appears to display insufficient specificity to justify its use.

Finally, we explored the Sc1472 antibody, raised against the C-terminus of human β_3-adrenoceptors. This antibody detected a band at the level 44 kDa, which was more prominent in extracts of β_3- than of β_1- or β_2-adrenoceptor-expressing cells and a diffuse band in the 60-80 kDa region that was largely absent from the lanes containing human β_1- or β_2-adrenoceptors. The 60-80 kDa band most probably reflects heterogeneity of the N-linked complex and carbohydrate moiety located close to the N-terminus of the receptor (Stiles et al. 1984). As an additional model system we have used UROtsa cells for evaluation of this antibody, as those cells express β_3-adrenoceptor mRNA (Ochodnicky et al., 2012) and also functionally respond to the β_3-adrenoceptor agonists TAK677 and BRL37344 (Limberg et al. 2010). Bands at 44 kDa and 60 kDa were observed in UROtsa cells in our study, further indicating promise for the selectivity of the Sc1472 antibody. Encouraged by the above findings we have also tested Sc1472 for use in immunocytochemistry, using detection approaches based on enzyme-linked and on fluorescent secondary antibodies. As the outcome for immunocyto- and histochemistry experiments not only depends on the primary antibody but on the whole experimental procedure (Petrusz et al. 1976), a range of conditions was tested in initial experiments. The chosen conditions yielded a selective staining with the alkaline phosphatase-conjugated secondary antibody. When using the fluorescent secondary antibody, a similar degree of specificity for the β_3-adrenoceptor-expressing cells was obtained, but the staining appeared to be largely associated with the nucleus, which questions the usefulness of this approach. Nevertheless, the Sc1472 antibody appears promising for use in immunohistochemistry.

From the cluster of papers published in 2009 it had become clear that disappearance of signals with blocking peptide or the presence of a single lane in Western blots is insufficient to prove target selectivity of an antibody (Michel et al., 2009). Thus, the present data add to a growing list of receptor antibodies evaluated by harder criteria. Such evaluation for additional antibodies has included those against M_3 muscarinic receptors (Grol et al., 2011), 5-HT2c receptors (Anastasio et al., 2010), H_4 histamine receptors (Beermann et al., 2012), S1P2 sphingosine-1-phosphate receptors (Randriamboavonjy et al., 2009), gonadotropin releasing-hormine receptors (McFarlane et al., 2011) and free fatty acide receptors (Ichimura et al., 2009) and has yielded both positive and negative results.

CONCLUSIONS

In summary, none of the five commercially available antisera against human β_3-adrenoceptors exhibited convincingly high target selectivity in immunoblotting with CHO cell membranes as compared to cells expressing the other β-adrenoceptor subtypes; the Sc1472 antibody appeared least unsuitable. In contrast, previous findings from other investigators (Kullmann et al., 2011;

Limberg et al., 2010) as well as our data demonstrate that both the LSA4198 and the Sc1472 antibody are promising for immunohistochemical applications. As these two antibodies have been raised against distinct epitopes, i.e. N- and C-terminus of the receptor, staining of the same structures by both antibodies in immunohistochemistry experiments can be taken as strong evidence for indeed labeling the β_3-adrenoceptor. Thus, based on our present and previous findings of others (Kullmann et al., 2011; Limberg et al., 2010) we propose that parallel staining of human tissues with LSA4198 and Sc1472 should be performed; while discordant results with the two antibodies may not be easy to interpret, any structure consistently stained by both antibodies is likely to indeed represent β_3-adrenoceptors.

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